

MICROORGANISMS IN SOLID MATERIALS
TASK III:
RECOVERY LEVELS OF MICROBIAL ORGANISMS
INOCULATED INTO SOLID PROPELLANT SPECIMENS

(Mod. 1)

VOLUME V

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I. INTRODUCTION

The objective of Task III is to determine the minimum number of microorganisms that can be inoculated in solid propellant and subsequently detected by cultural techniques. This extinction value (minimum number of microorganisms allowing detection) defines the limit for detection of growth of the added inoculum. In Task IV experiments, selected levels of organisms will be added as inocula to solid propellants; sterilization procedures will be applied and the propellant will be subsequently cultured. The absence of growth of the added inoculum does not necessarily mean that no organisms survived the sterilization procedures. Rather, it indicates that fewer organisms survived than represented by the extinction value established in Task III. Obviously, the smaller the size of the inoculum that can be detected by growth in culture, the greater the degree of confidence one may place on the effectiveness of the sterilization procedures applied.

The methods for culturing the inoculated propellant was purposefully adapted for detecting the organism present in the inoculum. It must be emphasized that the conditions for assessing the presence of microorganisms in this task may not be ideally suited for the detection of all organisms.

During the course of work on this task it became apparent that certain modifications must be made in the recovery techniques used in earlier work in order to increase the sensitivity of detection of organisms. Certain inhibitory substances were present in the propellant that were not found in other solids that were studied and these inhibitors had to be neutralized before a maximum recovery efficiency could be obtained. In addition to the neutralization approach, improvements were made in the method of pulverizing the material and in the preparation of the media. All of these changes tend to optimize the results and as a result the efficiency of the recovery was substantially improved over the work done in previous studies.

The results of our work in Task III have also indicated that ultimately these basic recovery techniques can be refined and extended until they may ultimately reach an efficiency of less than one microorganism per cubic centimeter.

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II. METHODS

A. PREPARATION AND ASSAY OF INOCULUM

The organisms studied in Task III were Bacillus subtilis var. niger, Cl. sporogenes, Serratia marcescens, Ulocladium, and Staphylococcus epidermidis.

1. Bacillus subtilis and Serratia marcescens

These organisms were suspended in dilution vials and viability counts were performed following decimal dilution by plate counting techniques within three days of the time the samples were prepared for submission to JPL. The organisms were adjusted to yield final concentrations of 10^2 , 10^3 , 10^4 organisms per cm^3 propellant. The organisms were intimately intermixed with heat sterilized dry Celite (2-3 grams per each level of organism inoculum), and submitted to JPL for incorporation in propellant specimens.

2. Staphylococcus epidermidis

This organism was maintained on Trypticase Soy Agar (BBL). Inocula from these agar slants were made into Trypticase soy broth. During the period of accelerated growth (logarithmic phase) the organisms were recovered from the culture media by centrifugation. They were washed three times in sterile distilled water and resuspended in a small volume of sterile distilled water. The cells were assayed by decimal dilution and plating techniques utilizing Trypticase Soy Agar.

3. Clostridium sporogenes

The Clostridial spores were prepared by growing vegetative organisms in Brain Heart Infusion Broth (DIFCO). Calcium chloride was added as a supplement to the media at a final concentration of 1% to enhance sporulation. The organisms were harvested by centrifugation at approximately $1000 \times g$

and the sediment was examined microscopically to insure that they were in the form of spores. The supernatant fluid was discarded and the spores were washed three times by alternately centrifuging and resuspending in sterile distilled water. The washed resuspended cells were subjected to exposure to heat at 80°C for a period of 10 minutes to destroy surviving vegetative forms and to ensure that only the spores survive. Following this heat shock step, the cells were stored at 5°C until required as an inoculum. In order to provide the inoculum at specific levels for JPL, the cells were assayed by decimal dilution in anaerobic deep agar tubes and by the use of Brewer Anaerobic Agar (BBL) plates.

4. Ulocladium

The Ulocladium was grown on Sabouraud agar medium (DIFCO). The organisms were harvested at the end of the first week of growth in distilled water and were subjected to sonication in an Acoustica Associates Model DR 50 AH ultrasonic bath for a period of 20 minutes at 40 to 60 ma. at a power output which produced cavitation. Following sonication the cells were centrifuged at 1000 x g for 10 minutes, the supernatant fluid discarded and resuspended in sterile distilled water. This procedure was repeated twice. The cells were then resuspended in sterile distilled water in a sterile solution bottle and maintained at 5°C until required for use as inoculum. At the time of submission of the organism to JPL, plate counts on Sabouraud agar medium (DIFCO) were performed.

In all instances the assayed microorganisms were mixed in small volume with a proportionately greater volume of heat sterilized Celite (Johns-Manville). The total weight of the Celite used for each level of organism inoculum ranged between 2 and 3 grams. With the exception of the "mixed inoculum" all organisms were supplied to yield three levels of concentration 10^2 , 10^3 , $10^4/\text{cm}^3$ propellant. The mixed inoculum was supplied at levels to yield 10^1 , 10^2 , and 10^3 organism/ cm^3 propellant. The contents of the vials were added to 1000 grams of propellant formula by personnel at JPL.

The propellant is composed of:

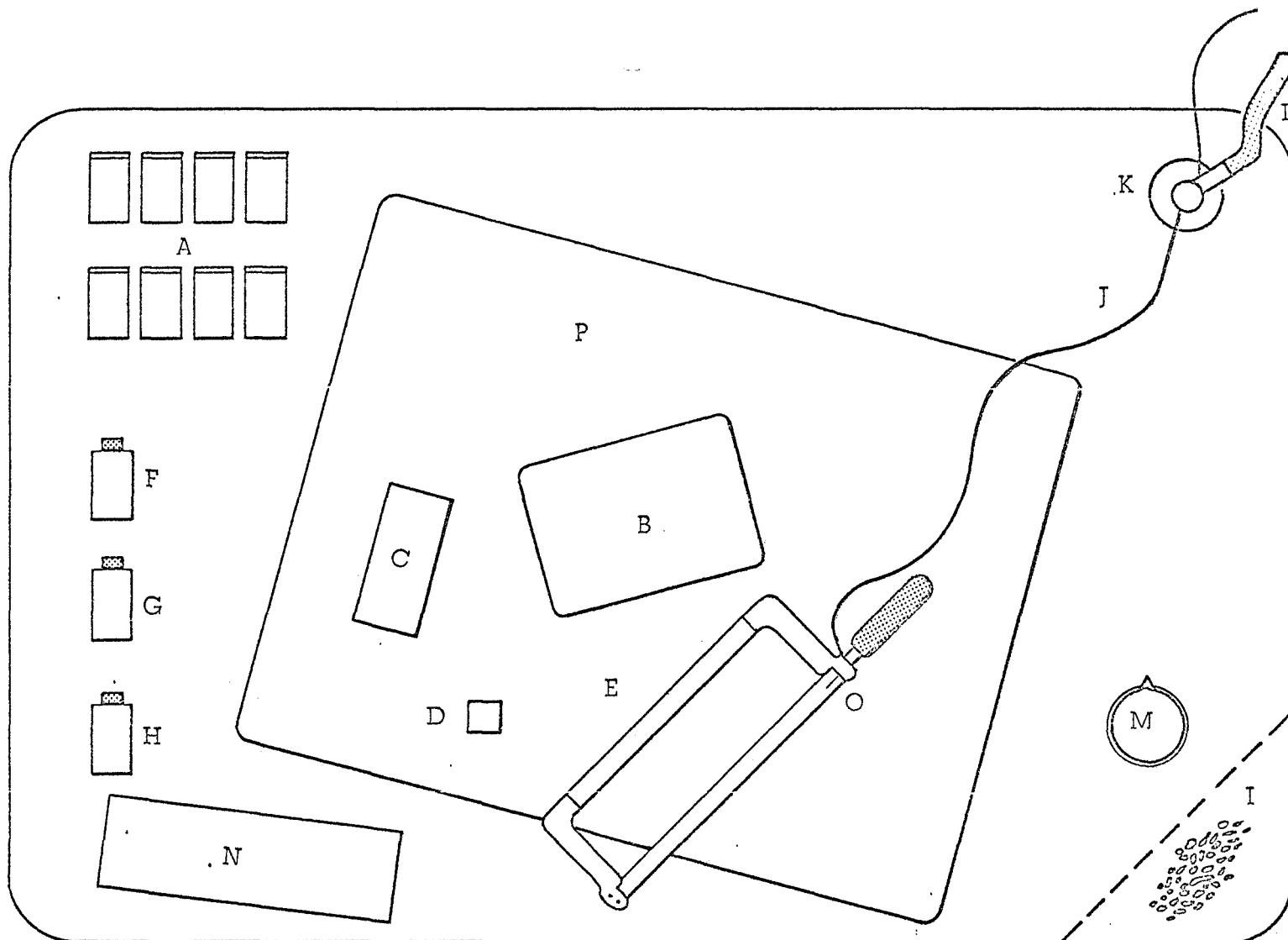
- 1) ammonium perchlorate
- 2) alroperse (reaction product of equal mixture of stearic acid and diethanolamine)
- 3) phenyl-beta-naphthylamine
- 4) 1, 1, 1-trimethylol propane
- 5) ferric acetylacetonate
- 6) 2, 4-toluene diisocyanate
- 7) poly propylene glycol
- 8) aluminum oxide

The propellant was returned to Dynamic Science as soon as possible following a 3 to 4 day curing period and immediately assayed for surviving organisms.

B. PULVERIZATION OF PROPELLANT

The propellant was enclosed in polyethylene sterilant bags (to liter capacity) as shown in Figure 1. These bags were equipped with a Teflon gland which permits the maintenance of a gas-tight system during the time period in which the contents of the bag are being sterilized. Each sterilant bag contained 60 to 70 ml of ethylene oxide (Matheson research grade), in a shallow dish. Several paper towels previously moistened with water were included in the bag to assure that humidity was near 50 percent. The effectiveness of ethylene oxide sterilization is markedly increased with elevated humidity. This atmosphere was maintained within the bag for a period of 12 to 16 hours to insure ample time for surface sterilization. The gland attachment, following sterilization, is subsequently attached to autoclave sterilized cotton packed exhaust tubing. The bag is evacuated using a water vacuum pump, after which the tubing is detached and sterile air is allowed to refill the bag and the bag is re-evacuated. Alternate exhaustion of the ethylene oxide from the bag and replacement with sterile air is thus accomplished. This procedure was repeated three times. Several dry paper

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LEGEND: A = Sterile water & culture media	F = 0.1 N NaOH	K = Gland
B = Wooden block	G = 0.1 N HCl	L = Bacteriological cotton utilized for evacuating bag
C = Propellant	H = Cysteine	M = Beaker cont. ethylene oxide
D = 5 ml beaker	I = Waste Cuttings	N = Paper towels (12)
E = Saw	J = Copper wire to ground	O = Thermistor

Figure 1. Sterile Polyethylene Enclosure

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towels were included in the sterilant bag to remove droplets of moisture from the internal surface of the bag. A segment of copper wire approximately 0.5 mm in diameter was attached through the conductive portion of the gland to a suitable ground. On the inner aspect of the bag the copper wire was attached to the blade of the saw. These precautions were taken to insure that static electrical discharge could not take place within the bag. Such discharge would be improbable since the humidity of the bag was maintained at or near 40 percent.

Previous studies on methods for pulverizing solid propellant resulted in limited and variable recoveries of organisms. The following techniques were evaluated in previous studies on solid propellant and found to be relatively ineffectual either in providing sufficient pulverization or in adversely affecting the organisms. These included the following techniques:

- 1) thin slicing with a blade-type microtome
- 2) drilling specimen propellant while submerged in culture media
- 3) abrasion with a "stickle back" rasp
- 4) abrasion with various types of files
- 5) homogenization in a Waring blender-type homogenizer

The use of saws of various conformations showed advantages over all previously attempted methods of pulverization. The blade found most serviceable was determined to contain 18 teeth per inch*. Several manufacturers provide such hacksaw blades. Some, however, contain toxic coatings which interfere with growth of microorganisms. A thermistor was attached to either the front one inch or the back one inch of the blade and a slow rate of sawing was used. The rate of sawing was so regulated that an increase in temperature of not more than 1°C resulted. Because the temperature at the saw tooth propellant interface may be several degrees higher than that indicated by the thermistor, the rate of sawing was adjusted to minimize possible thermal inactivation of organisms.

*Blade No. 1218-3, Clemson Bros., Inc.

The propellant was removed from the plastic container prior to placing it in the sterilant bag in order to facilitate surface sterilization of the propellant. Following the previously described sterilization step and the subsequent removal of the ethylene oxide from the bag, the propellant was scored to a depth of 2-4 mm. These cuttings were moved to a corner of the bag and sealed off. Previous tests had indicated that the outermost cuttings of the propellant were inhibitory to the growth of test organisms. The sampling areas of the propellant are shown in Figure 2. An attempt was made to sample at a random depth including sites near and distant from the surface. Roughly equivalent quantities were sampled from each of the indicated areas.

Beta propiolactone in place of ethylene oxide was used in 100 percent humidity for a 3-hour exposure period to induce surface sterilization. Our experience thus far indicates it offers the following advantages:

- 1) There is decreased permeation of sterilant through the interstices of the propellant.
- 2) Due to an approximate 20 fold increase in kill rate over ethylene oxide, shorter time periods are required to achieve surface sterilization. This also minimizes diffusion below the surface of the propellant.
- 3) The tendency for particles to agglomerate is decreased.

The particle distribution of the sawed propellant is indicated in Figure 3. Microscopically in all sizes of particles studied, innumerable fine cracks and checks were noted which likely have continuity with the interstices of even the largest particles. The larger particles appeared to result by fusion of smaller particles. This fusion effect could not be reversed by treatment with the Waring blender, sonication, or by treatment with wetting agents. Although Tween 80* was included in the culture media and did

*polyoxyethylene-sorbitan-monoaleate (Atlas Powder Co.)

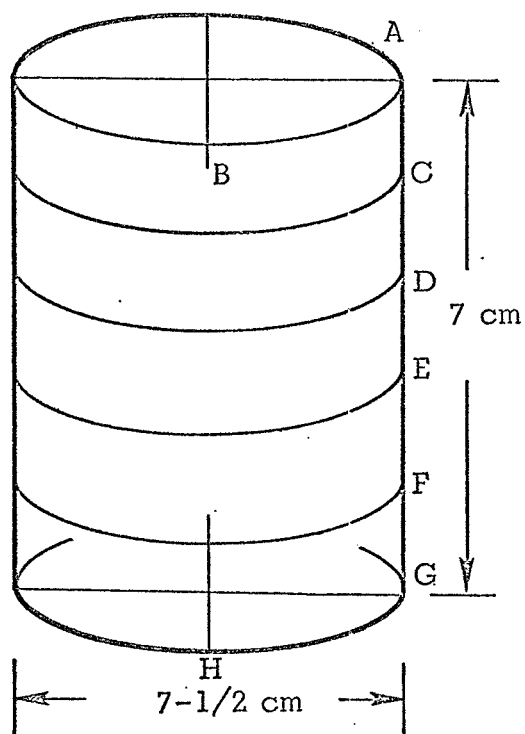


Figure 2. Sampling Areas Used For Solid Propellant

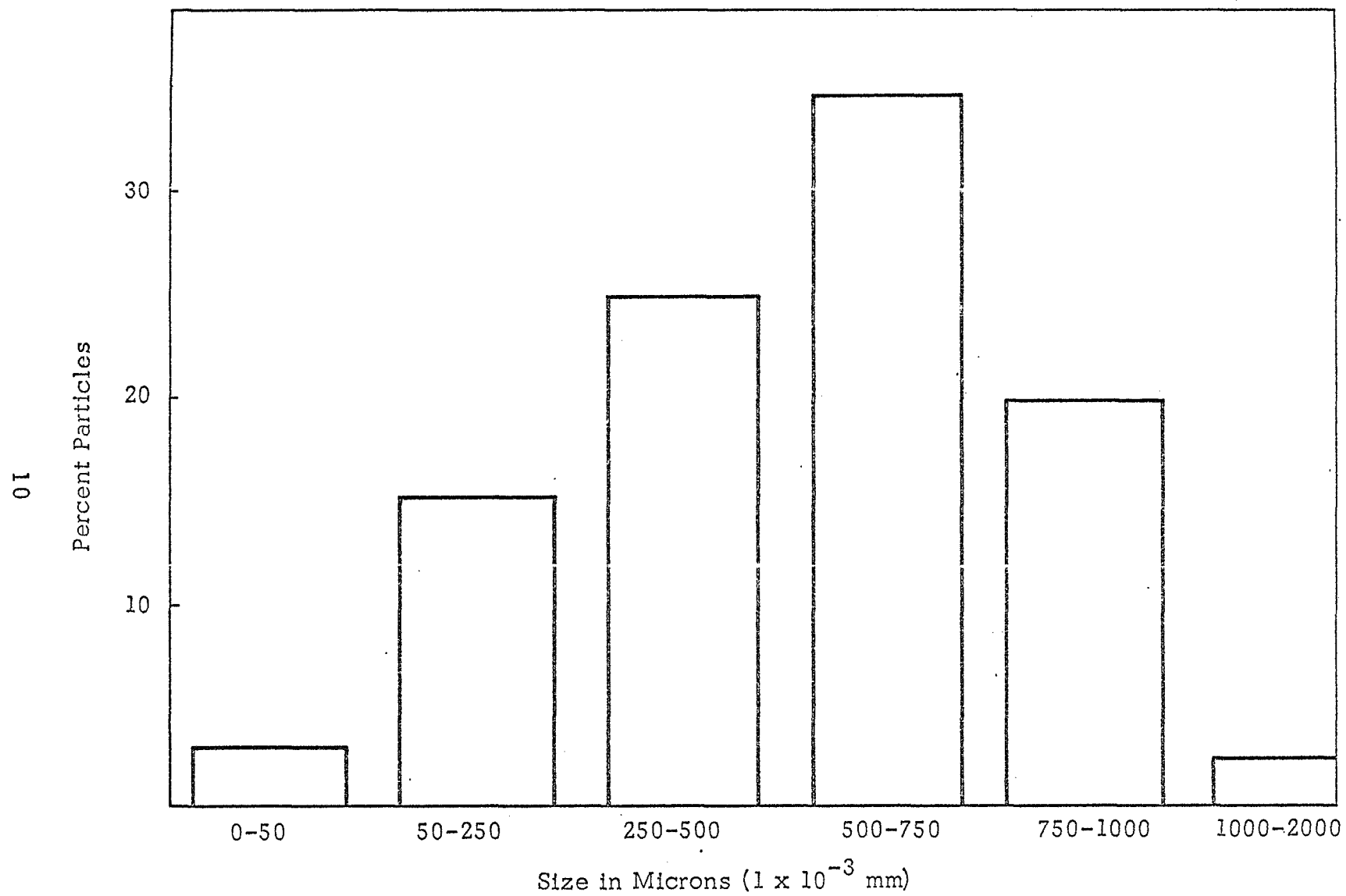


Figure 3. Size Distribution of Pulverized Propellant

provide superior recoveries (Figure 4), it does not appear that such recoveries were the result of inhibition of particle agglomeration.

The propellant particles obtained by sawing were placed in a 5 ml beaker. The beaker, when completely filled, contains 3.2 grams $\pm$ 200 mg. The quantities of propellant used in the culture studies consisted of multiples of 3.2 grams.

C. LEACHING OF PROPELLANT

The propellant while in the sterilant bag was added to a screw cap bottle containing 120 ml of distilled sterile water and a sterile Teflon coated magnetic stirring bar. The sterile screw cap containers were placed on a magnetic stirrer for 15 minutes. The supernatant fluid was treated as indicated in Figure 5. The redox potential of the leached propellant and the supernatant fluid was measured and this potential was adjusted to that considered optimum for each microorganism cultured. The pH was adjusted to 7.2 - 7.4 using sterile 1/10 normal acid or base except in the case of Ulocladium the pH was adjusted to 4.0 - 4.5.

The supernatant fluid was divided into three portions while in the sterile enclosure. One portion was centrifuged, stained with fluorescent dye, and examined microscopically for the presence of organisms. The second and third portions were added to culture media containing 1% Tween 80. The final volume of the leached supernatant fluid and culture medium was established by prior model experiments considering the inhibitory effect of the perchlorate from the propellant on the growth rate of the microorganism studied.

The ratio by weight of propellant to sterile distilled water which provided optimal recovery was determined to be 1:10. In previous studies, only the ability of a large inoculum of organisms to grow in the presence of the various propellant ingredients was determined. In the present study it was observed that concentrations of ammonium perchlorate as low as 0.5% interfered with the growth of some of the organisms.

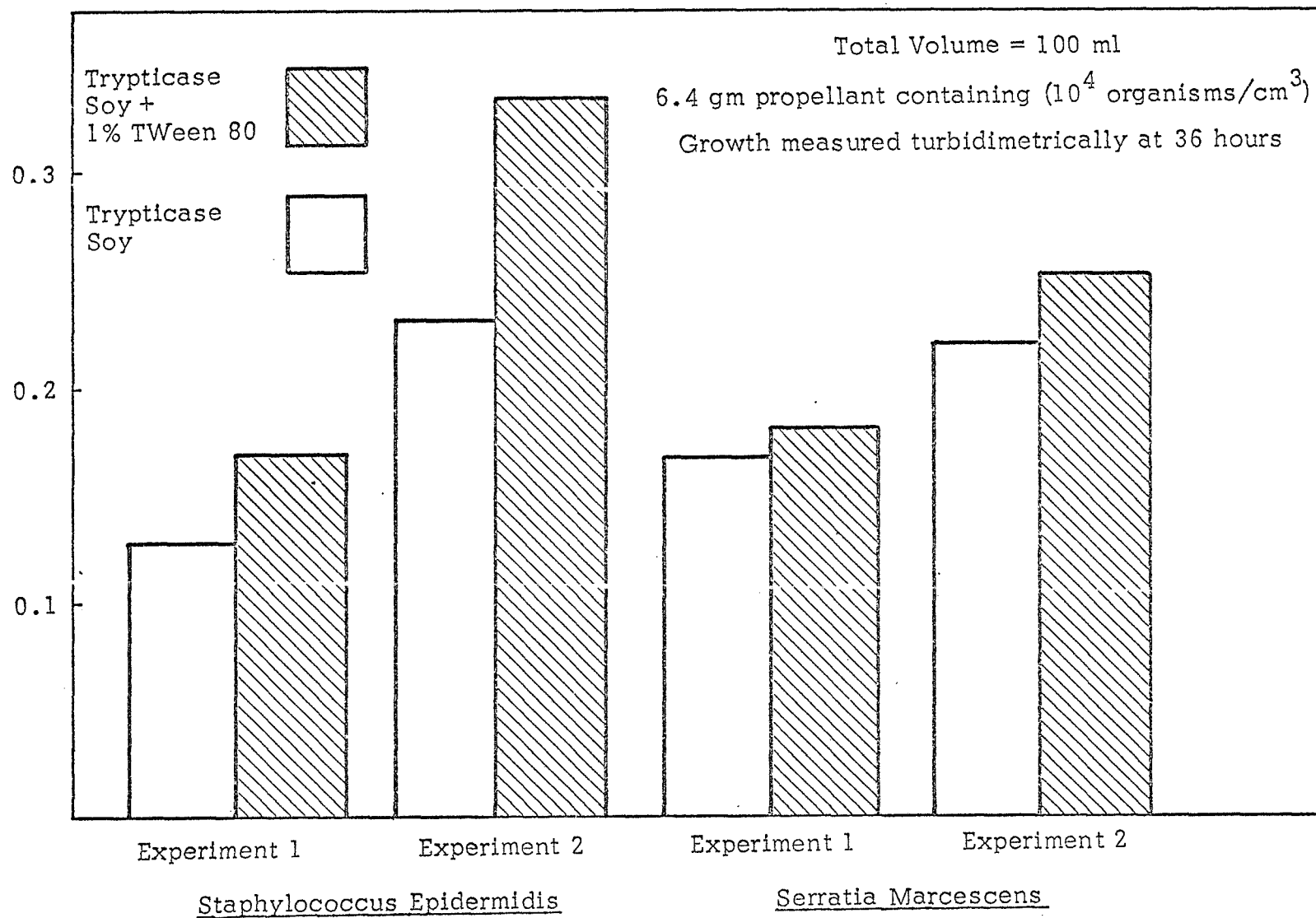


Figure 4. Effect of Tween 80 on Growth of Selected Bacteria
in Propellant as Determined Turbidimetrically

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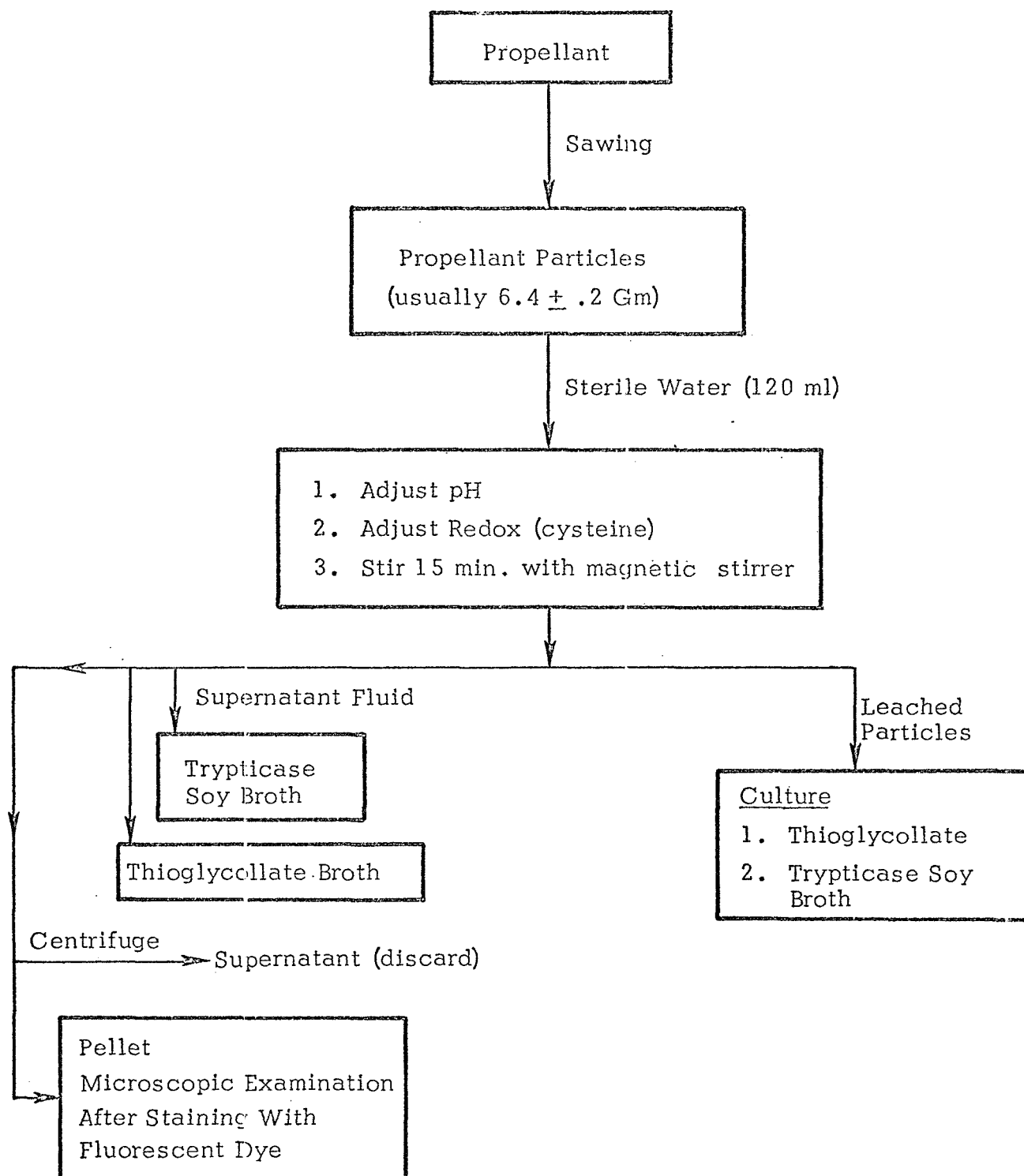


Figure 5. Summary Chart of Pulverization and Culture Methods

To the vessel containing the leached propellant an aliquot of cysteine was added to reduce the redox potential to the desired level. This level was previously arrived at by model experiments utilizing a sample of propellant outside of the bag. This provided a method for adding the required amount of reductant to the vessel while in a sterile environment.

In all cultural procedures routine controls were included for sterility of the enclosure's environment, the sterility of the growth media, and to insure the capability of the growth media to support the growth of low levels of microorganisms. Usually the latter control contained 10 and/or 10^2 levels of organisms. The temperature of incubation was maintained at 37°C . Additional cultures were maintained at 25°C for Ulocladium.

III. EXPERIMENTAL RESULTS

Recovery of test organisms was successful at 10^2 level of inoculum in all cases (Tables 1 - 6). B. subtilis, Cl. sporogenes and Staph epidermidis were recovered at 10^1 in the mixed inoculum (Table 6). Several contaminating types of organisms were recovered as well as the organism used in the inoculum. The time required for any particular culture to show evidence of bacterial or fungal growth varied considerably. The cultures containing organisms at the 10^4 level consistently showed evidence of growth prior to growth appearing in 10^3 , and growth was observed at 10^3 prior to 10^2 .

The organisms were identified by classical bacteriologic methods in order to verify that the growth observed in the culture vessels was due to the inoculum. In some instances growth due to the presence of contaminants in the propellant grew more rapidly than the inoculum. This was particularly true in some of the culture vessels containing the lowest level of inoculum. In some instances this made more difficult the task of recovery, and in some cases it may have obscured the recovery of the added microorganism.

Selected media and selective conditions were used in some experiments to inhibit the growth of contaminating organisms. These techniques were particularly useful when attempting to isolate Ulocladium. Although this fungal organism grows at a more rapid rate than many contaminating fungi, it does not grow as rapidly as many strains of microorganisms. By reducing the pH of the growth medium to 4.0 - 4.5 and utilizing a low ionic strength medium, profuse growth of Ulocladium was consistently observed under conditions which strongly inhibited the growth of contaminating bacteria.

Recovery of vegetative forms of Cl. sporogenes was poor in instances in which no attempt was made to counteract the strong oxidants present in the propellant. Following the addition of an optimal quantity of reducing agent and adjustment of the growth medium to the ideal redox potential

recovery of this organism was generally superior to recovery of any of the other inoculum tested.

The leached supernatant fluid consistently contained not only microorganisms used in the inoculum, but also contained variable quantities of contaminating microorganisms. Preliminary experiments have been carried out to determine the viability of these recovered organisms using other than cultural methods. Tetrazolium compounds in the presence of metabolically utilizable organic substrates lead in some instances to the appearance of reduced dye on the outer surface of microorganisms. Some of these organisms did not reduce the dye and therefore were likely either not viable or did not contain the enzymes which could be detected by the use of this dye. These experiments are at an early stage of study and do not represent a finalized experimental method.

TABLE 1

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: B. subtilis var. niger

	Average time (days) required for detectable growth			Sterility Control	Comments
	Inoculum Level/cm ³ Propellant				
	10 ²	10 ³	10 ⁴		
<u>Leached propellant particles</u>					
Trypticase soy broth	8I 4C	4I	2I	-	<u>Contaminants:</u> gm. + pos. cocci, gm. + rod central spore
Fluid thioglycollate	5I 2C	3I 4C	3I	-	
<u>Supernatant water extract</u>					
Trypticase soy broth	11I	7I 6C	4I	-	<u>Contaminants:</u> gm. + rod with granules, gram + rod with central spore, unidentified fungus
Fluid thioglycollate	12I 10C	7I	4I	-	
<u>Bacteriostasis control</u>					
Trypticase soy broth	4I	-	-	-	
Fluid thioglycollate	3I				
<u>Sterility control</u>					
Trypticase soy broth				0/4	
Fluid thioglycollate				0/4	

I = inoculum

C = contaminant

0 = no detectable growth

TABLE 2

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: Cl. sporogenes

	Average time (days) required for detectable growth			Sterility Control	Comments
	Inoculum Level/cm ³ Propellant				
	10 ²	10 ³	10 ⁴		
<u>Leached propellant particles</u>					
Anaerobic broth	4I 6C	3I	2I		<u>Contaminants:</u> gm. + rods with central spore small gram + rods without spores
Fluid thioglycollate	5I	5I 2C	5I		
<u>Supernatant water extract</u>					
Anaerobic broth	6I	5I	3I		Contaminants: gm. + rods with central spore
Fluid thioglycollate	0 3C	10 I	2I		
<u>Bacteriostasis control</u>					
Anaerobic broth	2I				
Fluid thioglycollate	3I				
<u>Sterility control</u>					
Anaerobic broth				0/4	
Fluid thioglycollate				0/4	

I = inoculum
 C = contaminant
 0 = no detectable growth

TABLE 3

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: Staphylococcus epidermidis (α)

	Average time (days) required for detectable growth			Sterility Control	Comments
	Inoculum Level/cm ³ Propellant				
	10 ²	10 ³	10 ⁴		
<u>Leached propellant particles</u>					
Trypticase soy broth	1II	5I	2I		<u>Contaminants:</u> gm. - rod, short gm. + rod
Fluid thioglycollate	8I 6C	4I 2C	1I		
<u>Supernatant water extract</u>					
Trypticase soy broth	9I	4I	2I		<u>Contaminants:</u> gm. + rod with central spore
Fluid thioglycollate	0	5I 2C	3I		
<u>Bacteriostasis control</u>					
Trypticase soy broth	4I				
Fluid thioglycollate	4I				
<u>Sterility control</u>					
Trypticase soy broth				0/4	
Fluid thioglycollate				0/4	

I = inoculum

C = contaminant

0 = no detectable growth

TABLE 4

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: Serratia marcescens

	Average time (days) required for detectable growth			Sterility Control	Comments
	Inoculum Level/cm ³ Propellant				
	10 ²	10 ³	10 ⁴		
<u>Leached propellant particles</u>					
Trypticase soy broth	10I	5I 3C	1I		<u>Contaminants:</u> large gm pos. rods, gm pos. cocci
Fluid thioglycollate	6I 1C	4I 6C	3I		
<u>Supernatant water extract</u>					
Trypticase soy broth	7I 10C	6I	3I		Contaminants: gm + rods with central spores, gm negative rods
Fluid thioglycollate	0	6I 3C	2I		
<u>Bacteriostasis control</u>					
Trypticase soy broth	2I				
Fluid thioglycollate	2I				
<u>Sterility control</u>					
Trypticase soy broth				0/4	
Fluid thioglycollate				0/4	

I = inoculum
 C = contaminant
 0 = no detectable growth

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TABLE 5

RECOVERY LEVEL OF INOCULATED PROPELLANT

Organism: Ulocladium

	Average time (days) required for detectable growth			Sterility control	Comments
	Inoculum Level/cm ³ Propellant				
	10 ²	10 ³	10 ⁴		
<u>Leached Propellant particles</u>					
Trypticase soy broth	9I 5C	6I	3I		<u>Contaminant:</u> mold not <u>Ulocladium</u>
Sabouraud broth	12I	5I 10C	5I		
<u>Supernatant water extract</u>					
Trypticase soy broth	0 7C		4I		<u>Contaminant:</u> gm pos. rod central spore
Sabouraud broth	16I		6I		
<u>Bacteriostasis control</u>					
Trypticase soy broth	3	-	-		
Sabouraud broth	4	-	-		
<u>Sterility control</u>					
Trypticase soy broth				0/4	
Sabouraud broth				0/4	

I = inoculum
C = contaminant
0 = no detectable growth

TABLE 6

RECOVERY LEVEL OF INOCULATED PROPELLANT

Mixed Inoculum Consisting of:

B = Bacillus subtilis, Ser = Serratia marcescens, U = UlocladiumCs = Cl. sporogenes, S = Staphylococcus epidermidis

	Average Time (days) required for detectable growth			Sterility Control
	Inoculum level/cm ³ Propellant			
	10 ¹	10 ²	10 ³	
<u>Leached propellant particles</u>				
Trypticase soy broth	B10, S11	B8, Ser 10, S9	B4, Ser 6, S5	
Fluid thioglycollate	Cs12	Cs6, B10, Ser9,S9	B5, Cs3,Ser 5, S10	
Sarbouraud broth (pH 4-4, 5)		U11, B14	U7, B16	
<u>Supernatant water extract</u>				
Trypticase soy broth	B12, S14	B7, Ser 12, S9	B3, Ser 7, S 5, U 17	
Fluid thioglycollate	Cs11	Cs9, B 17, Ser 12	Cs2, B7, Ser6, U14	
Sarbouraud broth (pH 4-4,5)		U 15	U10, B9, S16	
<u>Bacteriostasis control</u>				
Trypticase soy broth	B5, Ser4, S8, U12			
Fluid thioglycollate	Cs8, B6,S12,Ser14			
Sarbouraud broth (pH 4-4,5)	U10			
<u>Sterility control</u>				
Trypticase soy broth				0/4
Fluid thioglycollate				0/4

IV. DISCUSSION

Low levels of microorganisms $10^2 - 10^4/\text{cm}^3$ intentionally inoculated into solid propellants may be successfully detected by culture methods. The pulverization of the propellant by sawing has proven most useful in our studies. The pulverized samples obtained using this technique consistently give superior results to those obtained previously using other methods. Intermittent stirring with a magnetic stirrer aided the maintenance of aeration and also likely exposes new surfaces containing organisms.

Inhibitory substances were present in the propellant which interfered with the growth of all strains of microorganisms tested. It was essential that these inhibitory substances be removed from the solid propellant particles by leaching. One of the inhibitors present in dilute form in the leached fluid is ammonium perchlorate. The perchlorate may be reduced chemically by treatment with dilute strong reducing agents. The use of such agents had led to better recovery of microorganisms from both the leaching fluid and the pulverized propellant. The control of the reduction-oxidation potential of the growth medium may be as important as control of pH in the recovery of small numbers of microorganisms using culturing methods. Other inhibitory or potentially inhibitory substances include phenyl-beta naphthylamine, 1, 1, 1-trimethyl propane, ferric acetylacetonate, and 2, 4-toluene diisocyanate which are all constituents of solid propellants. In the present study there were no attempts to discern the order or magnitude of inhibition inducible by these substances, however, it is obvious that such experiments should be performed.

The use of fluorescent dyes in detecting the presence of organisms in the leached supernatant shows promise. This method is somewhat deficient in that there is no means of determining viability of organisms visualized with this technique.

Preliminary experiments with tetrazolium compounds and oxidizable organic substrates have proven promising. It has been possible to detect the presence of reduced dye on the surface of organisms leached from propellant and it is probable that this method will be extremely useful in propellant sterilization studies.

In a large percentage of the samples of propellant studied we have isolated microorganisms which were not present in the inoculum and which apparently represent JPL environmental contamination. This is to be expected since in the course of Task III investigations, no attempt has been made to provide the propellant in a state of sterility at the time the inoculum was incorporated into it.

V. SUMMARY AND RECOMMENDATIONS

In the course of the studies conducted in Task III of this program, we have detected microorganisms in inoculated solid propellant at all levels studied (10^2 , 10^3 , 10^4) and in some instances at $10^1/\text{cm}^3$. The presence of contaminating microorganisms in a high percentage of the propellant samples indicates that as future studies progress, preliminary steps may have to be taken to reduce the population of non-inoculated organisms. We have found that through the use of an improved means of pulverizing the propellant, control of pH, neutralization of toxic substances, and adjustment of redox potential it has been possible to materially improve detection of small numbers of organisms by culture. There are indicated areas of further improving the efficiency of culturing techniques for recovery of organisms from propellant. These include a more detailed study of the effects of certain constituents of the propellant on the test microorganisms, determination of optimum pH, and optimum conditions of electrostatic repulsion between propellant fragments and microorganisms.

The addition of neutralizers to growth media should be investigated. These neutralizers may reduce or reverse the effect of the constituents of the propellant on the microorganisms. This approach has been found to be useful in studies on the recovery of microorganisms previously exposed to hexachlorophene, organo-metallic compounds and quaternary ammonia compounds. Such neutralizers to counteract constituents of propellant may be incorporated into the growth media.

It is also possible that an excess of one or more growth factors may be required for the recovery of microorganisms inhibited by propellant constituents. The effect of an excess of such growth factors on growth should be systematically investigated. Two additional factors are important in the recovery of organisms from propellant. These include 1) the incorporation of high buffer capacity for organisms which produce organic acid and whose growth is inhibited by these acids, and 2) media of low ionic strength for the detection of contaminating organisms which are adversely affected by ionic strength.

It is apparent that with optimization of cultural techniques and the supplementation of other ancillary techniques that recoveries may be extended to less than 1 organism/cm³. The effects of the solid on the microorganism and/or the effects of pulverizing the solid material may preclude isolation of viable microorganisms by cultural methods. However, the enzymic activities of potentially viable organisms could provide a sensitive means of showing the presence of organisms which may still be in the living state. The physical and chemical conditions which induce sterility usually will destroy enzymic systems within the cell. For example, treatment with heat in sufficient quantity to cause sterilization will also cause denaturation of the protein moiety of the enzymes. Similarly, heavy metals, formaldehyde, phenol and similar agents cause concurrent sterilization and enzyme destruction.

One broad category of enzymic reactions which may be sufficiently sensitive include those which cause reduction of tetrazolium. The various substituted tetrazolium salts are characterized by water solubility and lack of visible color in the oxidized state and lack of water solubility and the development of intense color in the reduced state. Depending upon the nature of the substituents on the tetrazolium the color of the reduced dye may range from red to blue. Because of the change in phase of the reduced dye, it is possible to concentrate the dye following a prolonged exposure to the material containing organisms or organism fragments. An immiscible solvent could be added to the aqueous phase in small quantity to dissolve the reduced dye and at the same time concentrate it for photometric measurement if desired. Alternatively it would be possible with the light microscope to examine the particles produced by pulverization of the solid after incubation with the oxidizable organic substrate and the tetrazolium. In this case, the organisms and the organism fragments would be characteristically stained whereas the devitalized or nonviable organisms lacking the enzymic activities would not be stained by the dye.

Other enzyme assays which may be utilized for the detection of potentially viable organisms include cytochrome oxidase (using para-phenylene diamine which is converted to an intense purple color in the reduced state), peroxidase, and the reduction of NAD (nicotinamide adenine dinucleotide). The latter substances have a characteristic spectrum in the reduced state in the ultraviolet, but can also be measured more sensitively with fluorescence.

Solids can be treated with a fluorescent stain which will adhere strongly to all cell walls of microorganisms and may be so selected to fluoresce in a different region of the spectrum from the fluorescence (when present) characteristic of the solid itself. Some of this work has been reported in this task. Fragments of all cell walls of microorganisms will show intensely and can be easily detected by scanning methods using the fluorescence microscope. The principal areas that will require study include the suppression of interfering auto-fluorescent substances in certain solids and possibly the requirement for removing or destroying quenching substances in the mixture.